

# UNCLASSIFIED

AD NUMBER
ADB281661
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jul 2001. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Ft. Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 8 Jan 2003

THIS PAGE IS UNCLASSIFIED

AD \_\_\_\_\_

Award Number: DAMD17-98-1-8067

TITLE: Functional Analysis of SMAD Activation in TGF-B-mediated  
Negative Growth Control in Breast Epithelial Cells

PRINCIPAL INVESTIGATOR: Joshua P. Frederick  
Xiao-Fan Wang

CONTRACTING ORGANIZATION: Duke University  
Durham, North Carolina 27710

REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government  
agencies only (proprietary information, Jul 01). Other requests  
for this document shall be referred to U.S. Army Medical Research  
and Materiel Command, 504 Scott Street, Fort Detrick, Maryland  
21702-5012.

The views, opinions and/or findings contained in this report are  
those of the author(s) and should not be construed as an official  
Department of the Army position, policy or decision unless so  
designated by other documentation.

20020816 051

## NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

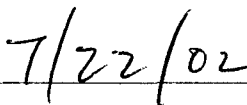
### LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8067  
Organization: Duke University

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

  
\_\_\_\_\_

  
\_\_\_\_\_

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> July 2001	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (16 Mar 98 - 15 Mar 01)	
<b>4. TITLE AND SUBTITLE</b> Functional Analysis of SMAD Activation in TGF-B-mediated Negative Growth Control in Breast Epithelial Cells			<b>5. FUNDING NUMBERS</b> DAMD17-98-1-8067	
<b>6. AUTHOR(S)</b> Joshua P. Frederick Xiao-Fan Wang				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Duke University Durham, North Carolina 27710  E-mail: jpf1@duke.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b> The ability of transforming growth factor-beta (TGF- $\beta$ ) to potently suppress the proliferation of normal breast epithelial cells may be central to its putative role in tumor suppression of early stage breast cancers. Thus, the elucidation of the mechanisms by which TGF- $\beta$ is able to exert these effects is relevant to the further understanding of breast cancer initiation and possibly prevention. The purpose of these studies is to further define the role of the Smads as TGF- $\beta$ -activated transcriptional regulators with particular attention to target genes involved in cellular growth inhibition, and thus, genes potentially involved in the tumor suppression of early breast cancers. The proto-oncogene c-Myc is repressed by TGF- $\beta$ and this repression is paramount for the manifestation of TGF- $\beta$ mediated growth arrest of epithelial cells. We have shown that Smad3 is required for both TGF- $\beta$ induced c-Myc repression and subsequently growth arrest in epithelial cells. The transcriptional repression of c-Myc is dependent on Smad3 binding to a novel Smad binding element within the TGF- $\beta$ Inhibitory Element (TIE) of the c-Myc promoter, termed a Repressive Smad Binding Element (RSBE). In conclusion, we have established Smad3 as an essential component of TGF- $\beta$ induced growth inhibition and contributed to the understanding of how this growth arrest program is initiated.				
<b>14. SUBJECT TERMS</b> Breast cancer, Transforming growth factor-beta, Smads, Tumor Suppression, growth inhibition, c-Myc				<b>15. NUMBER OF PAGES</b> 30
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

## **Table of contents**

Front cover	
Standard Form 298	2
Table of Contents	3
Introduction	4
Body	5-10
Figures	11-22
Key Research Accomplishments	23
Reportable Outcomes	24
Conclusions	25-26
References	27-29

## Introduction

Transforming growth factor-beta (TGF- $\beta$ ) clearly plays a complex role in the physiology of mammary gland development and autocrine/paracrine homeostasis, and the pathophysiology of breast carcinogenesis. These global effects of TGF- $\beta$  activity are thought to largely stem from the ability of this hormone to modulate the transcriptional activity of a variety of target genes that are classically divided into two subsets: those involved in cell cycle regulation and those involved in extracellular matrix modulation. The ability of TGF- $\beta$  to potentially suppress the proliferation of normal breast epithelial cells may be central to its putative role in tumor suppression of early stage breast cancers. However, it is important to note that in later stage breast cancers, in which epithelial cells are often refractory to the antiproliferative effects of TGF- $\beta$ , the modulation of the extracellular matrix (ECM) by the TGF- $\beta$  signal may contribute to breast cancer progression. Thus, the elucidation of the mechanisms by which TGF- $\beta$  is able to exert these transcriptional effects, both activating and repressive transcriptional modulation, is of particular interest. The Smad family of proteins are the most well characterized mediators of the TGF- $\beta$  signal. Upon phosphorylation by the TGF- $\beta$  receptor complex, Smads 2 and 3 heterodimerize with the central partner of Smad signaling, Smad4, and shuttle to the nucleus. This induced nuclear translocation suggested a potential role for the Smad family of proteins in transcriptional regulation; a role that has been largely established from much research in the last five years. The goal of the original proposal was to further define the role of the Smads as transcriptional regulators with particular attention to target genes involved in cellular growth inhibition, and thus, genes potentially involved in the tumor suppression of early breast cancers. Although the involvement of the Smad pathway in the transcriptional activation of TGF- $\beta$  target genes has been well characterized, the role of Smads in TGF- $\beta$  mediated transcriptional repression is only now being uncovered in the completion of this study and others. The elucidation of the molecular mechanisms involved in TGF- $\beta$  mediated repression of the proto-oncogene c-Myc have become the primary focus of this study due to the essential role of this repression in the manifestation of TGF- $\beta$ 's antiproliferative effects. The completion of these studies provide further understanding of Smad mediated signaling with an emphasis on the role of these effector molecules in growth inhibition and breast tumor suppression, and thus potentially provide a theoretical framework from which new breast cancer therapeutics may be devised.

## Body

The first technical objective of the original proposal, to **determine the mechanism through which the Plasminogen Activator Inhibitor-1 (PAI-1) gene is regulated by Smad3/4 and/or Smad2/4**, was chosen because PAI-1 was a well established TGF- $\beta$  target gene that was shown to be synergistically activated by overexpressed Smad 3 and 4 in a transcriptional reporter assay (23). In addition, we recently demonstrated that a Smad3/4 complex could physically interact with specific DNA response elements in the PAI-1 promoter, and that Smad4 alone is able to directly a specific sequence of DNA, CAGACA (22). Thus, although PAI-1 is a major constituent of the ECM, and not necessarily a mediator of the TGF- $\beta$  antiproliferative signal, it was chosen as a model gene to study the transcriptional activity of the Smads. Although progress was made in the completion of the first technical objective, various other groups were able to publish their findings of this line of investigation first. Recently, four reports were published that defined Smad3/4 binding sites within the PAI-1 promoter, and characterized these sites as functionally essential in the TGF- $\beta$  mediated transactivation of PAI-1 promoter reporter constructs (5,8,18,20). We and others have shown that Smad 2 and 4 overexpression can also transactivate PAI-1 reporter constructs, however the mechanism by which this is accomplished seems to be distinct from that mediated by Smad3 and 4 and is currently unknown. We have been able to define a distinct region of the PAI-1 promoter through which Smad 2 and 4 are able to transactivate this gene, but have not been able to demonstrate direct binding of Smad2/4 to any promoter sequence (unpublished data). The following factors have lead to an emphasis on Smad 3 signaling in this proposal, as opposed to Smad 3 *and* 2 signaling: the progress made in defining Smad3/4 complexes in the mediation of TGF- $\beta$  transcriptional control, and the availability of Smad 3 null cells in our laboratory. Although random degenerative oligos were obtained to complete part C of the first technical objective, another group first published the completion of this study (23).

From these findings, those obtained from solving the crystal structure of Smad3 bound to DNA and those obtained from the characterization of responsive elements in the promoters of TGF- $\beta$  inducible genes, the Smad Binding Element (SBE) was defined as GTCT or its palandrome AGAC. (3,5,8,17,18,20,21,22,23). One such study that defined an SBE within the promoter of a TGF- $\beta$  activated gene in which I contributed, was the demonstration that Smad3 and 4 and the transcription factor family of proteins, AP-1, synergize in the transcriptional activation of the cJun promoter by TGF- $\beta$  (21, attached ref. 1). This study further developed the role of Smad 3/4 as transcriptional activators with the ability to coordinate its activity with that of other transcriptional factors, and provides a conceptual basis for how the TGF- $\beta$  signal can be integrated with other disparate signals in the transcriptional regulation of a specific gene. Furthermore, in collaboration with other members of our laboratory, we have demonstrated that this transcriptional synergy between Smads and AP-1 is likely through the direct physical interaction of Smads and AP-1 family members (10, attached ref. 2). Finally, we have shown that the TGF- $\beta$  induced phosphorylation of Smad3 facilitates its interaction with the transcriptional coactivators p300/Creb Binding Protein (CBP) (16, attached ref. 3). Thus, although the exact aims delineated in the original technical objective one were

largely completed by other groups, my efforts, and thus your funding, in these other related studies has contributed to a better understanding of how Smads transduce the extracellular TGF- $\beta$  signal into transcriptional activation of target genes.

As previously delineated, the original technical objectives two and three were combined into the current technical objective 2. The focus of the first subaim of technical objective 2 is **to identify/characterize Smad target genes involved in TGF- $\beta$  mediation growth regulation**. We have recently created a Smad3 null mouse from which we have generated Smad3 null fibroblast and epithelial cells. With these tools we have definitively shown that Smad3 plays an integral role in TGF- $\beta$  mediated growth arrest in fibroblasts and cells of epithelial and lymphoid origins (4, attached ref. 4 and unpublished data). Thus, although Smad2 and 4 have been characterized as *bonafide* tumor suppressors due to their characterized mutations in human cancers, it is conceivable that Smad3 could play a tumor suppressor role in its antiproliferative capacity. Technical objective 2 was to be carried out as described with the exception that primary fibroblasts and epithelial cells derived from our wildtype Smad3 animals will be used in the completion of these studies due to the fact that they should not harbor undefined mutations commonly generated in cell lines. We have established adenovirus constructs expressing Smads 2, 3 and 4, and have successfully infected primary epithelial cells. Using the aforementioned primary cell systems, as well as the Smad expressing adenovirus constructs, we made the following steps in the completion of technical objective 2 in the second year of funding.

We have found that one mechanism by which TGF- $\beta$  may inhibit the growth of primary fibroblasts is through the downregulation of the G1-phase type cyclin, cyclin D1 (data not shown). 24 hours of TGF- $\beta$  treatment results in the reduction of cyclin D1 protein levels, and it was demonstrated that this induced reduction of cyclin D1 is Smad3 dependent. Furthermore, the basal levels of cyclin D1 in Smad3 null cells is markedly increased compared to wildtype fibroblasts, which is possibly due to endogenous TGF- $\beta$  loss of function. It is currently not known whether TGF- $\beta$ /Smad mediated repression of cyclin D1 is a direct or indirect mechanism. Other previously identified, cell cycle related targets of TGF- $\beta$  signaling (eg. p15, p21, cdc25a) were unaltered in wildtype cells, and thus were of no interest to pursue in this cell system (data not shown).

Additionally, primary epithelial cells were isolated from neonatal epidermis, as epithelial cells are a more appropriate cell type in which to study the TGF- $\beta$  antiproliferative signal. Wildtype epithelial cells are growth inhibited upon TGF- $\beta$  treatment 86% compared to only 35% in Smad3 null cells (Figure 1D). In these cells the c-Myc oncoprotein is repressed at both mRNA and protein levels by a Smad3-dependent mechanism (Figure 1A, B, C). Furthermore, adenovirus mediated overexpression of Smad3 and Smad3/4 are sufficient for repression of c-Myc mRNA (Figure 2).

Having determined that Smads had a direct role in TGF- $\beta$  induced repression of c-Myc, we chose to focus much of our efforts on the elucidation of the molecular mechanisms involved in this repression. We felt this shift in focus of the study was warranted due to the following rationale. First, the inappropriate overexpression of c-Myc has been reported in approximately 80% of breast cancers, and likely represents a relatively common event in early breast cancer formation (2,6,11). Second, the downregulation of c-Myc is essential to TGF- $\beta$  mediated antiproliferative effects in

epithelial cells and most likely an initiating event in this cellular process (31,32). Finally, it has been hypothesized that the tumor suppressor ability of the TGF- $\beta$  pathway may stem largely from its antiproliferative effects. Thus, we felt the elucidation of the mechanism involved in Smad mediated repression of c-Myc may yield valuable insight into the pathogenesis of early stage breast cancers. Specifically, we hoped to shed light on how TGF- $\beta$  exerts its antiproliferative effects in non-cancerous cells and on what changes may occur in cancerous cells to circumvent these antiproliferative effects and thus the tumor suppressive capacity of the TGF- $\beta$  pathway.

The role of the Smads in the transcriptional activation of genes activated by TGF- $\beta$ , such as PAI-1, has been well characterized, yet prior to this study, the Smads had not been previously implicated in the repression of genes repressed by TGF- $\beta$ , such as c-Myc. Indeed, our data from the second year of funding provided evidence for Smad involvement in TGF- $\beta$  mediated repression of c-Myc, however the exact nature of this role was unknown. Moses et al. discovered over 10 years ago that a sequence within the c-Myc promoter, termed the TGF- $\beta$  Inhibitory Element (TIE), was required for TGF- $\beta$  mediated transcription repression of the c-Myc gene (33, 34). It was recently reported that an endogenous Smad-containing complex formed directly or indirectly on the previously identified TIE of the c-Myc promoter in response to TGF- $\beta$  treatment (7). Massague et al. made the important discovery that the TGF- $\beta$  induced recruitment of Smads to Smad Binding Elements (SBE), thus SBE-dependent gene activation; and TIE-recruitment, thus TIE-dependent repression are separable events. It was shown that ras and erbB2 overexpression in breast cancer epithelial cells was able to abrogate TGF- $\beta$  induced recruitment of Smads to the c-Myc TIE, c-Myc repression and growth inhibition, yet did not affect Smad:SBE binding. (*Ibid.*) Given these results, we sought to determine the nature of Smad:TIE binding and further elucidate the mechanism of Smad-mediated repression of c-Myc.

As reported in the second annual report, we showed that Smad3 was essential and Smad3/4 overexpression was sufficient for c-Myc mRNA repression (Figures 1 and 2). We were able to further demonstrate that loss of Smad3 in primary colon epithelium resulted in elevated levels of c-Myc protein (Figure 3A). The elevated levels of c-Myc may be a direct consequence of the absence of the endogenous repressive signal of Smad3. To complement the data obtained from Smad3 wildtype and null keratinocytes, we also found that TGF- $\beta$  mediated repression of c-Myc RNA was abrogated in a TGF- $\beta$  responsive keratinocyte line, HaCat cells, that were stably transfected by a Smad3 dominant negative (S3 DN) expression construct (Figure 3B).

We next sought to determine how Smads 3/4 were involved in TGF- $\beta$  induced transcriptional repression of c-Myc. As first reported by Massague et al., a TGF- $\beta$  inducible complex containing Smad3 and 4 was recruited to exogenous SBE and TIE DNA sequence in a DNA precipitation (DNAP) assay (Figure 4) (7). However, the question remained how Smads were recruited to the TIE sequence. Was the inducible Smad:TIE interaction direct or was it mediated through another unidentified transcription factor that directly bound to the TIE sequence? We were able to show that the Smad3:TIE interaction was direct in an electrophoretic mobility shift assay (EMSA) in which purified recombinant protein was incubated with radiolabeled DNA probe. The recombinant Mad Homology Domain 1 (MH1) of Smad3 bound to radiolabeled SBE with higher affinity than corresponding recombinant MH1 preparations of Smads 1 and 2,

as reported, yet a similar binding specificity to TIE probe was also observed (Figure 5A). In addition, a similar relative affinity between Smad3:SBE and Smad3:TIE was revealed (Figure 5B and 5C). Thus, Smad3 is capable of specific and direct binding to the TIE sequence with a similar relative affinity as to the previously characterized Smad Binding Element. In an attempt to differentiate Smad:SBE from Smad:TIE interactions a Smad3 MH1 mutant (R75A) was generated and employed in similar EMSAs. The R75A mutant was chosen as a direct interaction of R75 of Smad3 with the guanine of the SBE GTCT was revealed in the crystal structure of Smad3:SBE (3), and thus it was hypothesized that this mutation would be deficient at least in SBE binding. As seen in Figure 6A, the R75A Smad3 mutant was deficient in SBE binding, yet was also deficient in TIE binding. This does not necessarily suggest that the same amino acids of Smad3 that mediate the SBE interaction also mediate the TIE interaction as it is possible that this mutation may disrupt proximal structure of Smad3. We were able to demonstrate a difference in Smad binding to these two DNA sequences from the finding that recombinant full length Smad4 was only able to directly interact with SBE, yet not TIE sequence (Figure 6B).

We next sought to further dissect which nucleotides within the TIE were important for Smad3 binding. Was the SBE-like CAGA sequence within the TIE sequence, or a novel DNA element that would define a distinct Smad binding sequence responsible for mediating the observed Smad3 interaction? We generated scanning mutations across the TIE, as defined in the Figure 7 legend, to unveil important nucleotides in the Smad3:TIE interaction employing both cold competition EMSAs and biotinylated DNA precipitations (DNAPs). The critical sequence for Smad binding within the TIE is maximally comprised of TGGCGGA, which is distinct from the previously defined SBE GTCT or AGAC (Figure 7A and B). The importance of this sequence to Smad3 binding was supported from a methylation interference assay from which it is demonstrated that methylation of the bold G's in TGGCGGA interfered with Smad3 binding (Figure 7C). Thus, we have characterized a novel Smad binding element that we have tentatively termed a Repressive Smad Binding Element (RSBE).

The sequence that was characterized as essential for mediating Smad3 binding to the TIE is also essential for TGF- $\beta$  mediated repression of the c-Myc promoter. The wildtype (WT) and corresponding mutant (M1-M8) c-Myc promoters were cloned into a luciferase reporter vector and employed in transient transfection, luciferase assays to measure TGF- $\beta$  mediated repression. (Figure 8A) It was further shown that the isolated WT TIE sequence is both sufficient and required for TGF- $\beta$  mediated repression (Figure 8B).

The functional analysis of the c-Myc promoter represented in Figure 8 showed that an overlapping sequence within the TIE was important for not only TGF- $\beta$  mediated repression, but also basal activity of the c-Myc promoter. This suggested the possibility that an activating transcription factor was recruited to an overlapping if not identical sequence that is important for TGF- $\beta$  induced Smad recruitment to the TIE. Indeed, the serum response of the isolated WT TIE reporter was dependent on the nucleotides previously determined essential for Smad binding (data not shown). Due to the sequence similarity of the TIE to a consensus E2F activating transcription factor binding site, we determined if E2F proteins were capable of binding this site in DNAPs and EMSAs. As shown in figure 9A, E2F4 is capable of binding exogenous biotinylated TIE sequence, but not SBE sequence. E2F4 seems to bind a closely overlapping sequence to the defined

RSBE. Mutations M5-M6 within the TIE seem to disrupt E2F4 binding, whereas mutations M4-M6 abrogate Smad3 binding in DNAPs (Figures 9A and 7B). It is of particular interest that TGF- $\beta$  treatment results in inducible binding of Smad3 to the TIE, whereas E2F-4 binding is diminished in the presence of TGF- $\beta$  (Figure 9A). We thus hypothesized that TGF- $\beta$  treatment results in a competition between a repressive Smad3 and an activating E2F4 for binding to the TIE. A purified recombinant preparation of the DNA binding domain of E2F4 (E2F4DBD) is capable of binding TIE probe albeit with lower relative affinity compared to the Smad3 MH1:TIE interaction (Figure 9B). Furthermore, the demonstration that the addition of recombinant Smad3 MH1 results in diminished E2F4DBD binding to the TIE (Figure 9B). This *in vitro* result lends credence to our hypothesis that Smad3 competes with E2F4 binding to the TIE. For a summary of this hypothesis see Figure 10.

In order to definitively understand the molecular mechanisms involved in TGF- $\beta$  mediated repression of the c-Myc gene and elucidate what proteins are bound to the TIE within the c-Myc promoter *in vivo*, we have performed chromatin immunoprecipitation (ChIP) assays. For the first time that we are aware, we have been able to demonstrate TGF- $\beta$  induced binding of Smad3 to elements within the promoters of TGF- $\beta$  target genes *in vivo*. TGF- $\beta$  treatment results in the specific binding of Smad3 to both the SBEs within the PAI-1 promoter and the TIE within the c-Myc promoter (data not shown). This data is not represented in a figure as it is currently in preliminary form. We have been able to repetitively demonstrate *in vivo* Smad3 binding to the promoters of PAI-1 and c-Myc, yet are experiencing high background levels specifically with the amplification of the c-Myc promoter yet not with the amplification of the PAI-1 and negative control  $\beta$ -actin promoters. We are currently attempting to rectify this background problem to complete this portion of the study for publication.

The original technical objective 3, to **identify new target genes regulated by the Smads**, was to be carried out as part of the modified technical objective 2. However, this objective was to be accomplished with a different technical methodology than that originally proposed, i.e. the SAGE (Sequential Analysis of Gene Expression) system. Due to the availability of Smad3 null cells, these cells in conjunction with wildtype counterparts were to be used to identify new target genes regulated by Smad3. Isolated mRNAs from wildtype and Smad3 null cells treated in the absence and presence of TGF- $\beta$  were to be analyzed with a commercially available microarray system pending additional funds. During the third year of funding, we became aware of a similar study that was completed by a different research group. Considering this possible duplication of research efforts coupled with financial and time constraints we decided that our efforts were better spent on other aspects of this proposal.

The third task was to **define the *in vivo* role of Smad3 in mouse models of breast cancer**. The availability of the Smad3 null animals in our laboratory provides an extremely valuable tool to address this line of investigation. In preliminary histological analysis of Smad3 null mammary ductal epithelial, there has been no overt evidence of hyperplasia. We had planned to study role of Smad3 deletion in two mouse models of breast carcinogenesis: 7,12-dimethylbenz-[a]-anthracene (DMBA) and APCmin mouse induced mammary tumorigenesis. Due to the increased focus that was given to the identification of c-Myc as a direct target for Smad mediated transcriptional repression

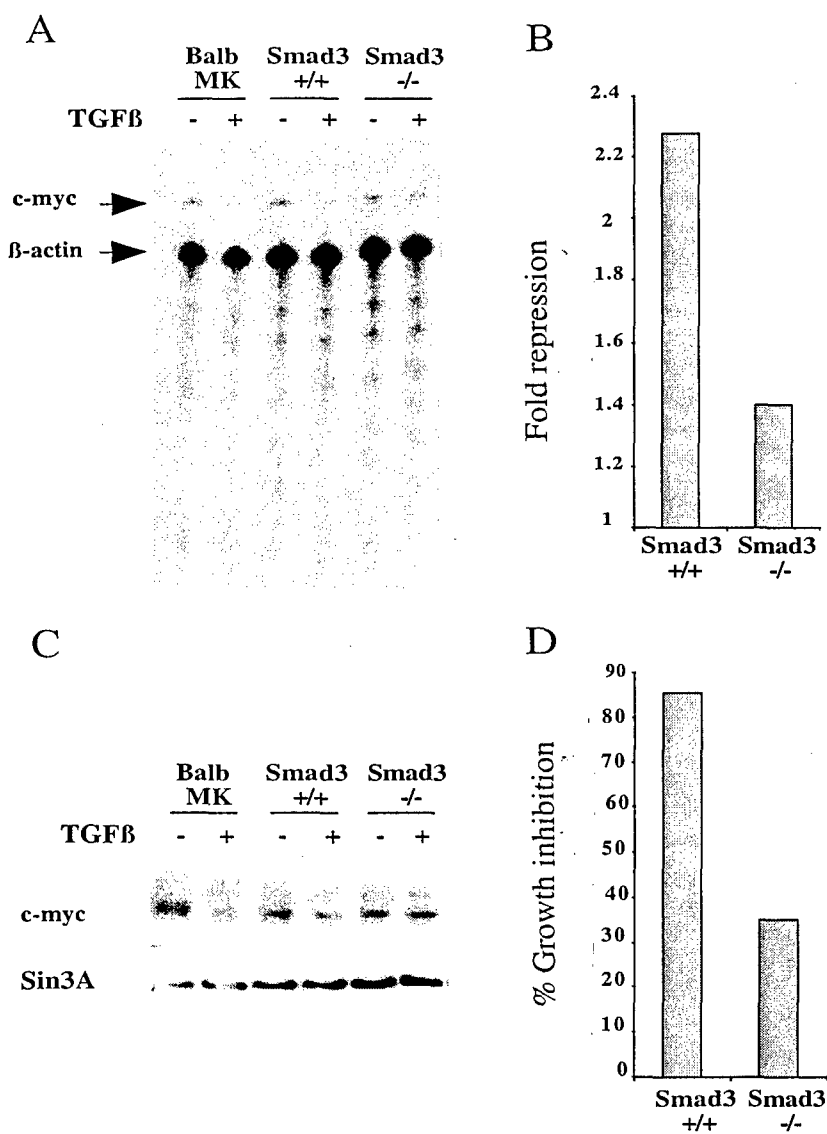
and the analysis of TGF- $\beta$  induced repression of c-Myc, we were unable to make significant progress in the completion of the third task. However, we did backcross the Smad3 targeted deletion over eleven generations into the pure strain of APCmin mouse that yields a breast cancer phenotype, i.e. APC min c57B6.

The APCmin mouse harbors an inactivating APC mutation and APC heterozygote mice develop colonic adenomas with high penetrance, and mammary tumorigenesis with lower incidence (12). APC is a tumor suppressor protein that functions in the *wnt* signaling pathway as a negative regulator. It serves to sequester  $\beta$ -catenin in the cytoplasm, and targets this transcriptional activator for degradation. With a positive *wnt* signal, or inappropriate activation of this pathway in a cancerous cell,  $\beta$ -catenin translocates to the nucleus to form a functional transcriptional complex with the DNA-binding protein tcf-4 (13).

The *wnt* and TGF- $\beta$  pathways have long been linked epigenetically in both antagonistic and synergistic interaction in developmental studies in *Xenopus* and *drosophila*. We have recent evidence that the *wnt*/APC/ $\beta$ -catenin/tcf-4 pathway and the TGF- $\beta$ /Smad3 pathway may be antagonistic on the molecular level, and thus possibly on the more global level of cancer development. Smad3 was found to interact with a tcf family member in a yeast two-hybrid screen and directly interact with *in vitro* translated tcf-4 (data not shown). The relevance of this interaction is supported by the recent demonstration that Smad4,  $\beta$ -catenin and Lef1/tcf directly interact to synergistically activate a target gene involved in a developmental context (25). In the context of cancer, it was demonstrated that the TGF- $\beta$  and *wnt* pathways may act antagonistically as compound heterozygosity of APCmin and Smad4 results in enhanced malignancy of colorectal carcinoma (27). It was recently shown that cyclin D1 and c-Myc are direct target genes of  $\beta$ -catenin/tcf-4 transcriptional activation (26, 28, 29, 30). These studies in conjunction with our findings that these genes are repressed by TGF- $\beta$  in a Smad3 dependent manner raises the possibility that these pathways may directly antagonize the function of one another in the transcriptional regulation of c-Myc and cyclin D1. Although the inappropriate activation of this pathway is classically associated with colon cancer development and tcf-4 was first shown to be highly expressed only in colonic epithelium, a recent report demonstrates comparable levels of expression in mammary epithelium (1). Furthermore, the inappropriate activation of this *wnt* pathway has been linked to poor prognosis in certain breast cancers (26). It was thus hypothesized that this crossing of mice, which would harbor the oncogenic activation of the *wnt* pathway and the loss of the potentially tumor suppressive Smad3 pathway, would result in more severe breast and colon cancer formation than that seen in APC heterozygote animals alone. The molecular mechanisms involved in the potential antagonistic relationship between the TGF- $\beta$  and *wnt* pathways will be studied on a cellular and molecular level with the aid of cells isolated from the aforementioned mouse crossing, and retroviral constructs overexpressing  $\beta$ -catenin, tcf-4 and Smad proteins. These retrovirus constructs have been completed.

We have currently only performed very preliminary analysis of APCmin/Smad3null offspring resulting from the proposed crossing. There is a very early incidence of mortality in these animals with females being most severely affected. Another graduate student within our laboratory has adopted these studies.

## Figures



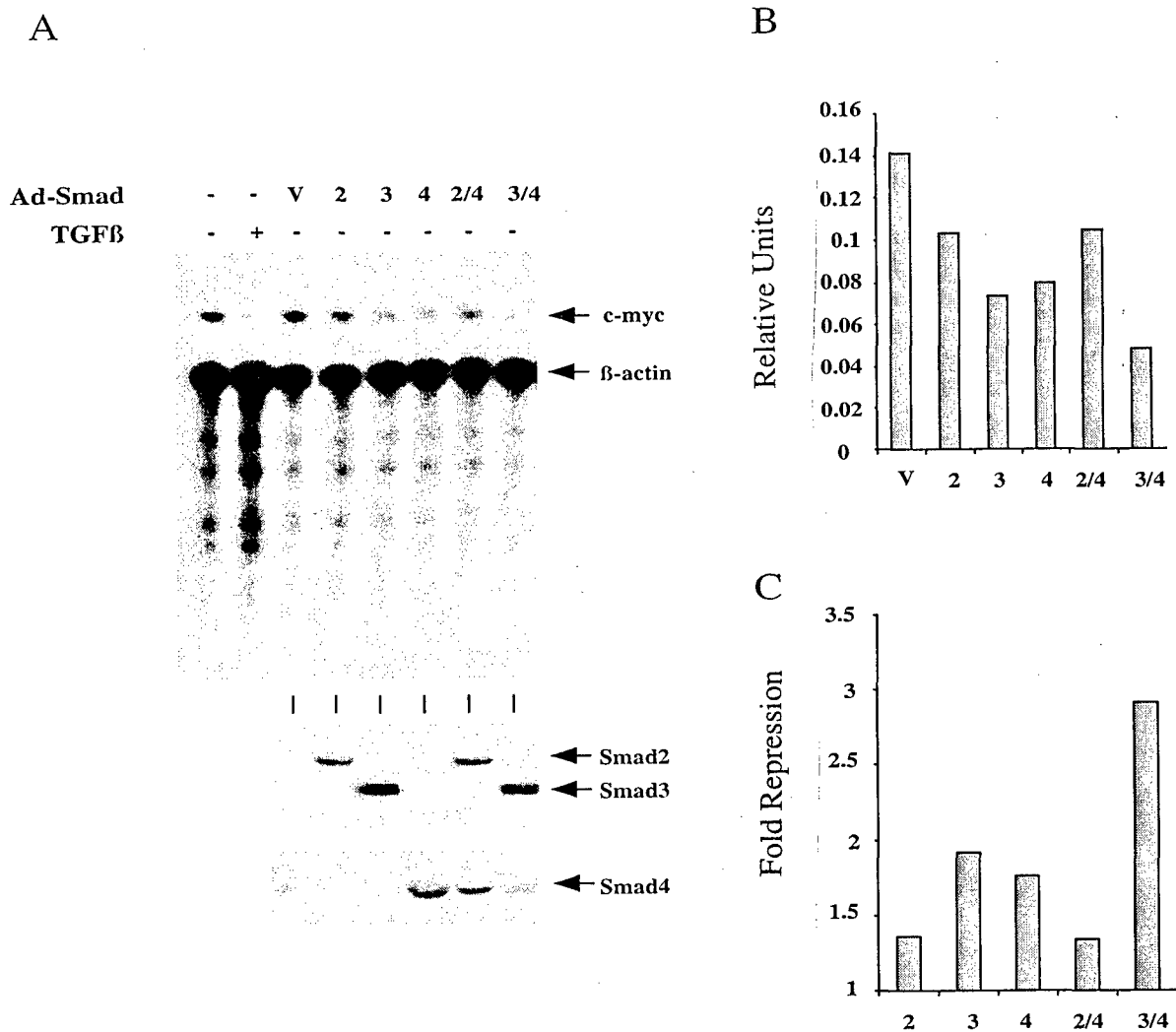
**Figure 1. Smad3 is critical in TGF- $\beta$  mediated repression of c-Myc and growth arrest of epithelial cells.**

(A) mRNA from wildtype and Smad3 null primary keratinocytes treated with 100 pM TGF- $\beta$  for one hour was isolated and analyzed by RNase protection with c-Myc and  $\beta$ -actin riboprobes.

(B) Densitometric analysis of (A) in which fold repression is graphed.

(C) 100  $\mu$ g nuclear lysate isolated from wildtype and Smad3 null primary keratinocytes were analyzed for c-Myc protein levels by Western. Sin3A Western analysis is shown as a loading control.

(D)  $^3$ H-thymidine incorporation assay of primary keratinocytes derived from wildtype and Smad3 null mice. Values are reported as a ratio of incorporated  $^3$ H-thymidine in the absence and presence of 100 pM TGF- $\beta$  treatment.



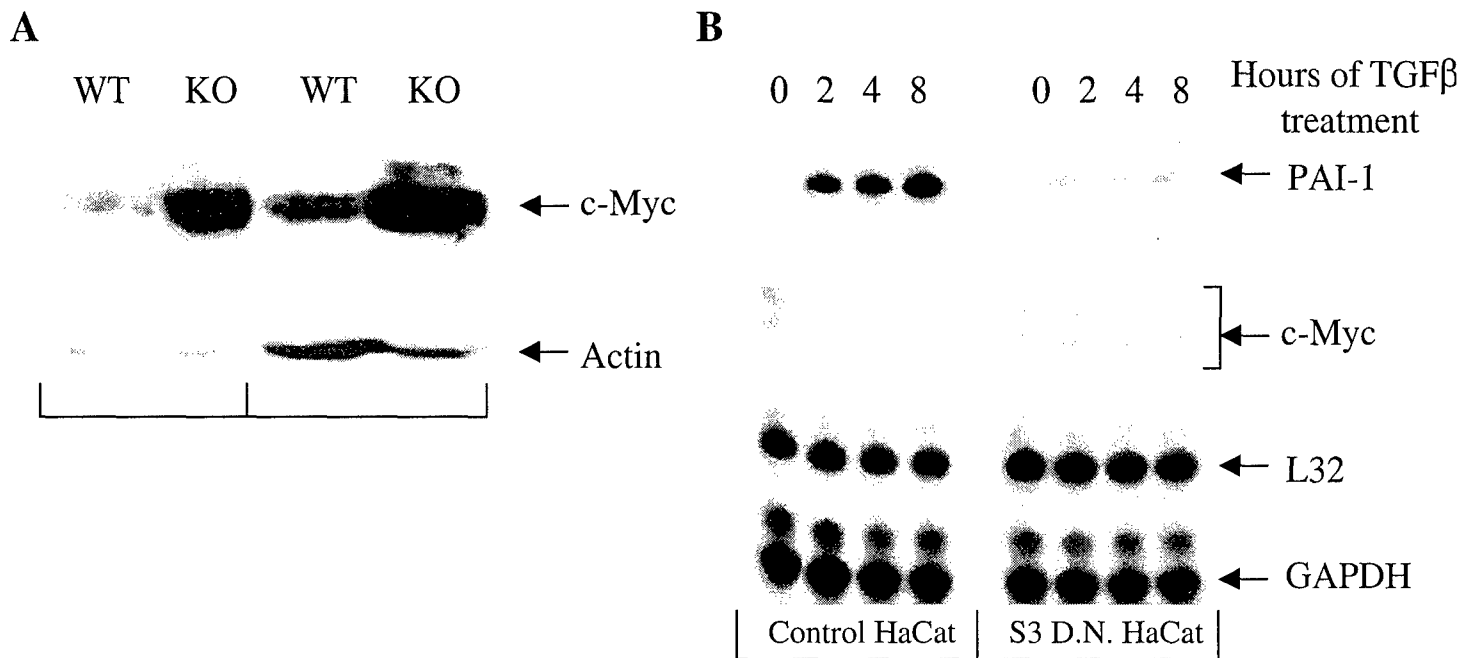
**Figure 2. Adenovirus-mediated expression of Smad 3 and 4 are sufficient for repression of c-Myc transcript levels.**

Mouse keratinocytes were treated with vehicle or 10 ng/ml TGF- $\beta$  for one hour or infected with adenoviruses carrying empty vector, Smad2, Smad3, Smad4 or co-infected with Smad2 and Smad4 or Smad3 and Smad4 at an M.O.I. of 100.

(A) 15  $\mu$ g of total mRNA was analyzed by RNase protection with c-Myc and  $\beta$ -actin riboprobes. Smad Western analysis of lysates from the infected cells are shown in the lower panels.

(B) Densitometric analyses of the RPA in (A).

(C) Data in (B) normalized to the vector control

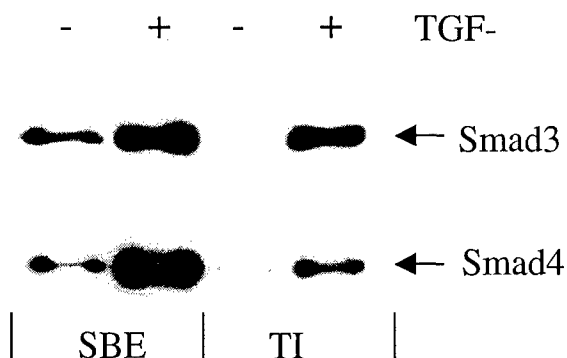


**Figure 3. Dysregulation of c-Myc in the absence of functional Smad3.**

(A) c-Myc protein levels are elevated in Smad3 null colon epithelium. Colon epithelium was isolated from Smad3 wildtype (WT) and null (KO) 2-3 month old mouse littermates of a mixed c57B6/129 background. Western analysis for c-Myc and actin, as a loading control, of 30  $\mu$ g protein are shown. Brackets indicate two individual sets of WT and KO littermates

(B) TGF- $\beta$  induced repression of c-Myc is abrogated in the presence of Smad3 dominant negative (S3 D.N.) over-expression. mRNA was isolated from control HaCat and Smad3 dominant negative HaCat cells treated with TGF- $\beta$  for 0, 2, 4 and 8 hours, as indicated. HaCat cells are spontaneously immortalized human keratinocytes. mRNA levels for PAI-1, a TGF- $\beta$  induced, SBE-activated gene and c-Myc were assessed by ribonuclease protection assay (RPA) on 5  $\mu$ g of total mRNA. L32 and GAPDH mRNA levels serve as loading control indicators.

**A**



**B**

SBE (16b.p.): 5'-AGTAT GTCT\*AGAC TGA-

TIE (30b.p.): 5'-TTCTCAGA GGCTTGGCGG GAAAAAGAACGG-

**Figure 4. Inducible binding of Smad 3 and 4 to both SBE and TIE DNA sequence upon TGF-β treatment.**

(A) 300 µg of whole cell lysate obtained from sonicated HaCat cells treated with vehicle or TGF-β for 1 hour was incubated with 1.54 µM of double-strand biotinylated SBE or TIE, as indicated. DNA-bound protein complexes were isolated by subsequent incubation with streptavidin-linked agarose beads, centrifugation and washing. DNA-bound protein was then separated by SDS-PAGE and analyzed by western for Smad3 and Smad4.

(B) The sequences for SBE and TIE employed for oligonucleotide precipitations and subsequent electrophoretic mobility-shift assays (EMSAs). The boxed SBE sequence indicates the previously characterized Smad Binding Element, whereas the boxed TIE sequence indicates the identified TGF-β Inhibitory Element within the c-Myc promoter.

**A**

No protein  
Smad1 MH1  
Smad2 MH1  
Smad3 MH1  
No protein  
Smad1 MH1  
Smad2 MH1  
Smad3 MH1

2x MH1 →

1x MH1 →

← 1x MH1

**B**

0 2.29 nM 556 nM  
Smad3 MH1  
0 2.29 nM 556 nM  
Smad3 MH1

15

SBE TIE SBE TIE

0 0.320 nM 700 nM  
Cold TIE  
0 0.320 nM 700 nM  
Cold SBE

**C**

Free TIE  
probe →

+ 15 nM Smad3 MH1

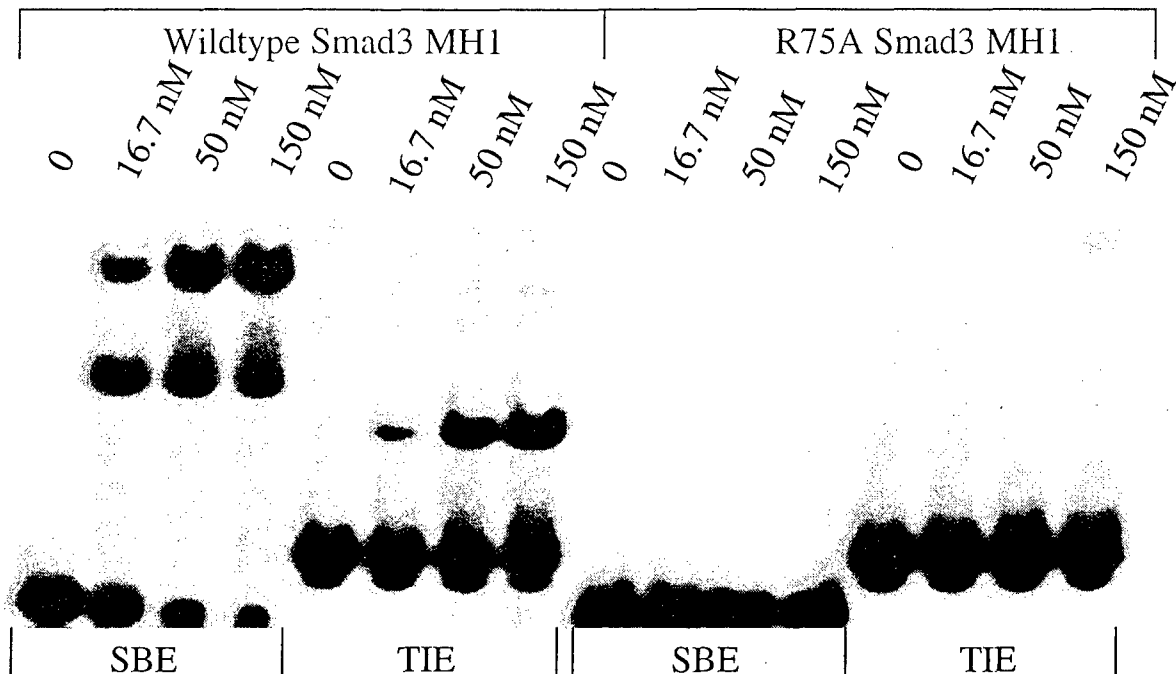
**Figure 5. The MH1 domain of Smad3 specifically binds to the c-Myc TIE with similar relative affinity as to the SBE.**

(A) 15 nM of recombinant Smad1, Smad2 and Smad3 MH1 domains were individually incubated with radiolabeled SBE and TIE in a EMSA. Two Smad3 MH1 complexes shift the SBE probe due to the palindromic nature of the SBE probe employed. One molecule of the MH1 domain bound to one molecule of SBE is represented by 1x MH1, whereas two molecules of the MH1 domain bound to one molecule of DNA is represented by 2x MH1. Only one complex is shifted with Smad3 MH1 domain:TIE incubation.

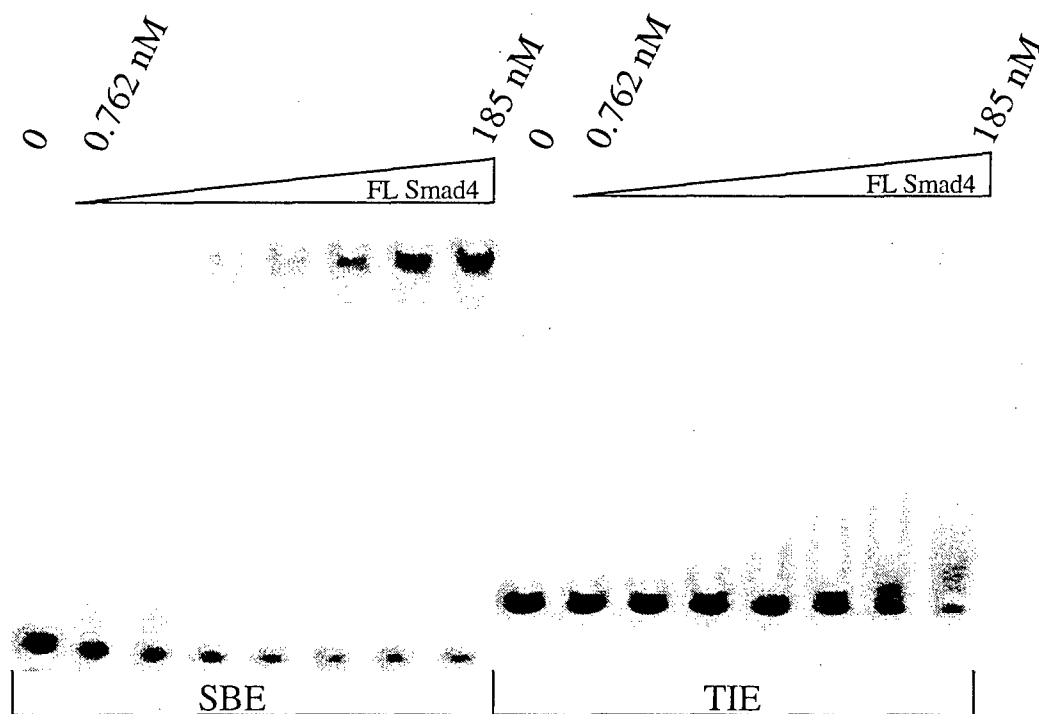
(B) The relative affinities of the Smad3 MH1:SBE and Smad3:TIE interactions were determined by EMSAs, in which the indicated concentrations of Smad3 MH1 recombinant protein was incubated with a fixed amount of radiolabeled probe. Three fold dilutions of protein, from 556 nM to 2.29 nM, were used.

(C) Similar relative affinities was confirmed by a DNA cold competitor EMSA. 15 nM of Smad3 MH1 domain recombinant protein was incubated with radiolabeled TIE and increasing amounts of cold competitor DNA. The mobility shifts with cold TIE are shown on the left, whereas those with cold SBE are on the right. Three fold dilutions of DNA, from 700 nM to 0.320 nM, were used.

A



B



**Figure 6. Binding of full length (FL) Smad4 to the SBE, but not the TIE demonstrates distinct protein recognition by the two DNA sequences.**

(A) An EMSA was performed similar to that in Figure 5 (B), except wildtype Smad3 MH1 domain was used in the mobility shifts on the left whereas a mutant Smad3, R75A, was incubated with the radiolabeled probe on the right. The R75A mutant Smad3 was created for this purpose as this arginine was shown by X-ray crystallography to directly interact with the guanine in the GTCT that constitutes the SBE.

(B) A similar EMSA was performed with increasing concentrations of full length Smad4 incubated with radiolabeled SBE shown on the left and TIE on the right. Three fold dilutions of protein, from 185 nM to 0.762 nM, were used in this assay.

A

+ 700 nM TIE

cold competitor::

WT	M1	M2	M3	M4	M5	M6	M7	M8	
0	WT	M1	M2	M3	M4	M5	M6	M7	M8

+ 15 nM  
S3 MH1

Free WT  
TIE probe

WT TIE:	5'-TTC TCA GAG GCT TGG CGG	GAA AAA GAA CGG-3'
M1 TIE:	5'-TTC TCG TAG GCT TGG CGG	GAA AAA GAA CGG-3'
M2 TIE:	5'-TTC TCA GCT GCT TGG CGG	GAA AAA GAA CGG-3'
M3 TIE:	5'-TTC TCA GAG ATT TGG CGG	GAA AAA GAA CGG-3'
M4 TIE:	5'-TTC TCA GAG GCC GGG CGG	GAA AAA GAA CGG-3'
M5 TIE:	5'-TTC TCA GAG GCT TAT CGG	GAA AAA GAA CGG-3'
M6 TIE:	5'-TTC TCA GAG GCT TGG ATG	GAA AAA GAA CGG-3'
M7 TIE:	5'-TTC TCA GAG GCT TGG CGA	TAA AAA GAA CGG-3'
M8 TIE:	5'-TTC TCA GAG GCT TGG CGG	GCT AAA GAA CGG-3'



TGF- $\beta$ :

Smad3 ↑

Smad4 

	M6
	M5
	M4
	M3
WT	

U

Free + Smad3  
Free

3' G C A A

G A A A

AG

AG

10

C

٢٥

ע א

51

L

F

I

5

5

)

G

△

U

5

2

7

Previously defined TI

# RSBE

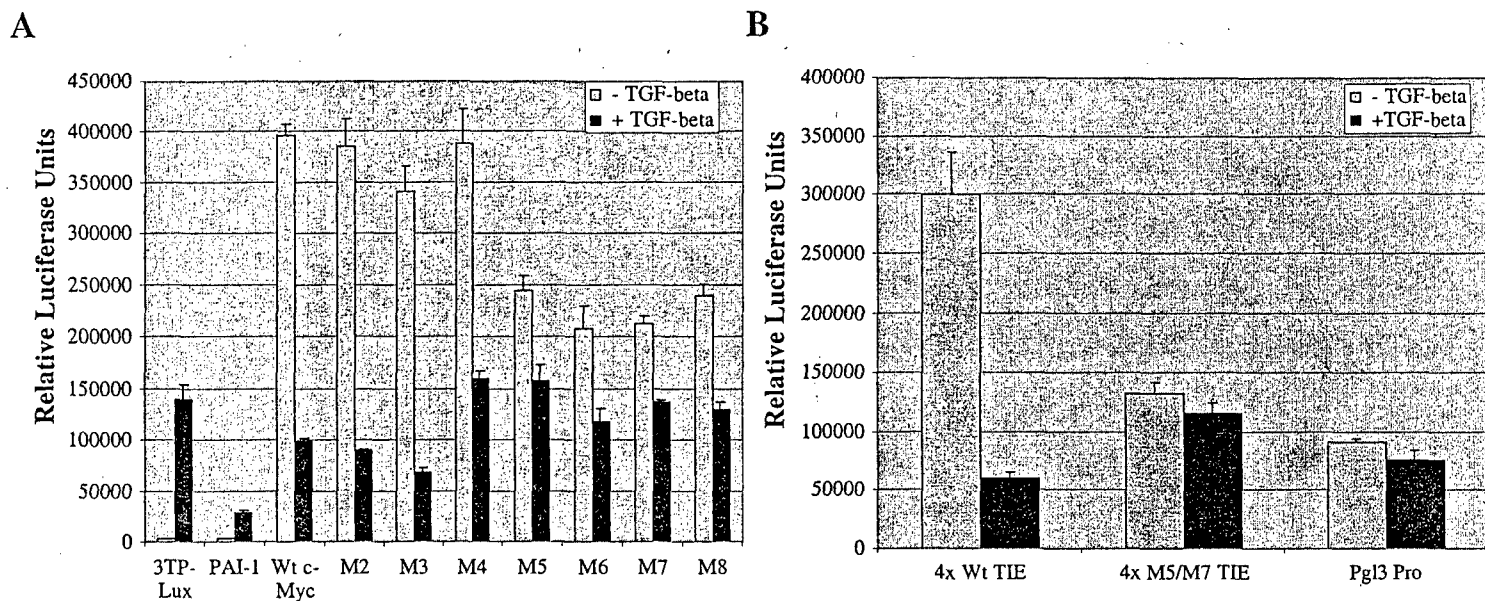
81

**Figure 7. Smad3 binds to a core sequence within the TIE that is distinct from the SBE. This sequence maximally comprised of TGGCGGGA has been termed a Repressive Smad Binding Element (RSBE).**

(A) A DNA cold competitor EMSA similar to that described in Figure 5 (C) was performed to determine the Smad3 binding sequence. 15 nM of recombinant Smad3 MH1 domain was incubated with radiolabeled wildtype TIE and 700 nM of cold TIE competitor. The sequence for the wildtype (WT) TIE and various, scanning TIE mutants (M1-M8) is listed. The number of asterix indicates the importance of the two base pairs that were mutated in mediating Smad3 binding.

(B) DNA precipitations as described in Figure 4 (A) define the same Smad binding sequence within the TIE as above. Biotinylated wildtype TIE and TIE mutants M3-M6 were used in this experiment.

(C) The guanines GGCGG within the TIE are shown to be important in mediating Smad3 binding by methylation interference assay. Wildtype TIE was radiolabeled on the 5' end of the sense strand of DNA, methylated with dimethyl sulfate and then incubated with recombinant Smad3 MH1 domain. The methylated DNA:protein mix was separated by PAGE and free, or unbound, probe as well as protein-shifted probe was isolated by electro-elution. The free and bound probes were then piperidine cleaved and separated by denaturing PAGE.

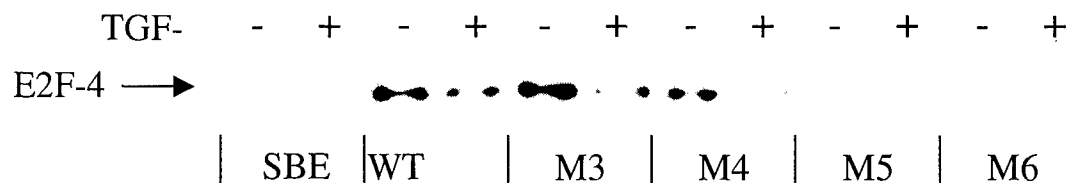


**Figure 8. The RSBE characterized by Smad-DNA interaction studies within the c-Myc promoter is required and sufficient for TGF- $\beta$  mediated transcriptional repression.**

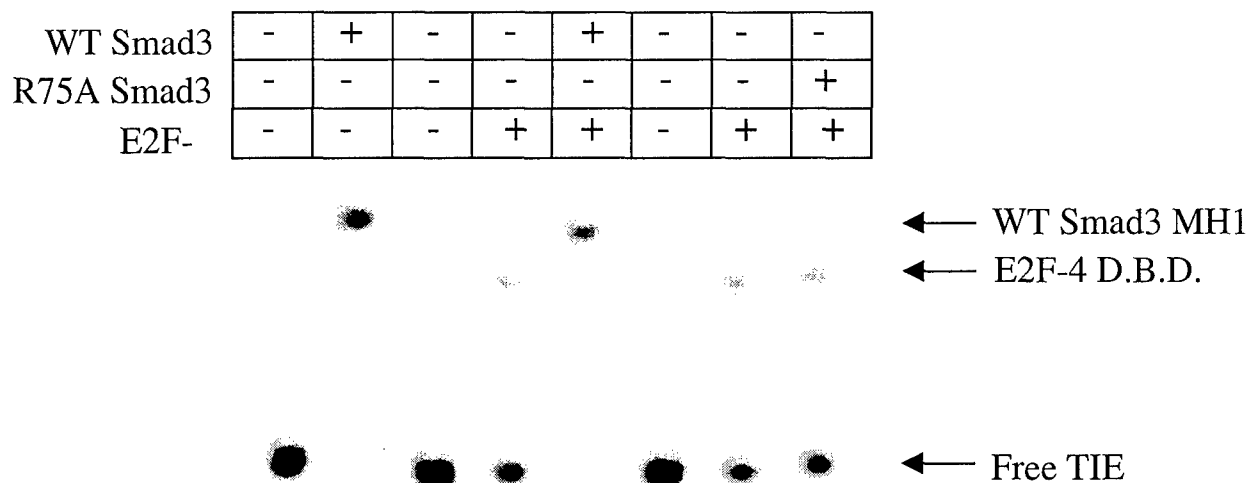
(A) HaCat cells were transiently transfected with equal amounts of the indicated promoter-driven luciferase reporters, and cultured in the absence and presence of 100 pM TGF- $\beta$  for 24 hours. Cells were then harvested and levels of luciferase measured by luminometer. 3TP-lux and PAI-1 are SBE-activated promoters. The WT c-Myc promoter was cloned into a reporter construct and mutations created (M2-M8) corresponding to those utilized in the characterization of the protein-DNA interaction.

(B) HaCats were transiently transfected as in (A) with luciferase reporter constructs containing four copies of the WT (4x WT TIE) or M5/M7 mutant (4x M5/M7 TIE) RSBEs cloned upstream of the SV40 promoter. Pgl3 Pro is the SV40 promoter-driven luciferase reporter vector.

**A**



**B**



**Figure 9. Specific binding to the c-Myc TIE/RSBE sequence by E2F-4 is competed against upon TGF- $\beta$  treatment and addition of recombinant Smad3.**

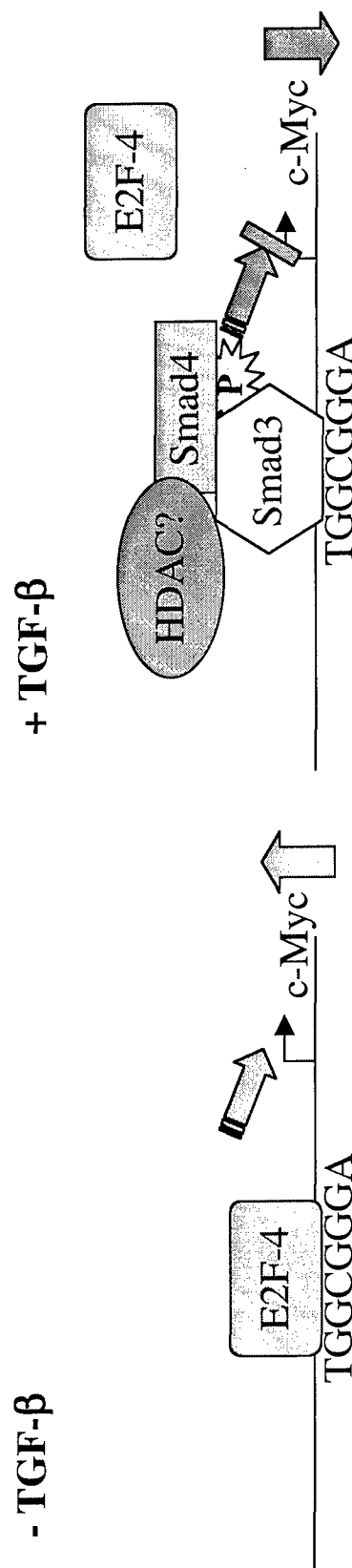
(A) Biotinylated oligonucleotide precipitations were performed as in Figure 1 (A). Biotinylated SBE, WT TIE, M3, M4, M5 and M6 as defined in Figure 7 (A) were used as indicated. Following SDS-PAGE, the presence of E2F-4 was determined by Western. The presence of the E2F family of transcription factors bound to this TIE/RSBE sequence was suspected due to sequence similarity to a consensus E2F binding site.

(B) 185 nM of recombinant wildtype, but not mutant R75A, Smad3 MH1 domain is able to compete with 556 nM of recombinant E2F-4 DNA-binding domain (D.B.D.) to radiolabeled TIE.

**Figure 10.**

### Mechanism?

The E2F transcription factor family member, E2F-4, is also capable of directly binding to the sequence within the c-Myc promoter we have termed the RSBE. The sequence to which E2F-4 binds seems to be overlapping if not the same as to which Smad3 is recruited upon TGF- $\beta$  treatment. In accordance with the finding that these two DNA-binding proteins seem to bind the same sequence, their binding to the RSBE seems to be mutually exclusive. In a population of asynchronously cycling keratinocytes, E2F-4 appears to be able to bind to the RSBE in a constitutive manner, at least in some stage of the cell cycle. However, upon TGF- $\beta$  treatment and subsequent recruitment of Smad3 to the RSBE, E2F-4 binding to this site is abrogated. It is our hypothesis that the binding of E2F transcription factors to this site is required for transcriptional activation and/or maintenance of c-Myc, and that upon TGF- $\beta$  exposure, Smad3 is able to perturb this E2F-mediated activity through competitive binding to the RSBE within the c-Myc promoter. Once Smad3 is bound to its repressive target sequence it may mediate active repression of c-Myc m-RNA levels via Smad recruitment of a histone deacetylase (HDAC) complex. Although we have not seen HDAC association with the RSBE upon TGF- $\beta$  treatment, the HDAC inhibitor trichostatin A (TSA) prevents TGF- $\beta$  induced repression of c-Myc mRNA levels (data not shown), thus suggesting a role for HDACs in this transcriptional repression.



## Key Research Accomplishments

- Smad3 and 4 and the transcription factor family of proteins, AP-1, synergize in the transcriptional activation of the cJun promoter by TGF- $\beta$ .
- Smads and the transcription factor family of proteins, AP-1, directly interact with one another.
- TGF- $\beta$  induced phosphorylation of Smad3 facilitates its interaction with the transcriptional coactivators p300/Creb Binding Protein (CBP).
- Smad3 is an integral component of TGF- $\beta$  mediated growth inhibition in primary epithelial cells and fibroblasts.
- Smad3 is essential for the TGF- $\beta$  mediated repression of cyclin D1 and c-Myc in primary cells.
- The loss of Smad3 in mouse colon epithelium results in elevated levels of the proto-oncogene, c-Myc, which may contribute to the occurrence of colonic adenocarcinoma formation reported in Smad3 null mice.
- Smad3/4 overexpression is sufficient to inhibit c-Myc mRNA levels.
- TGF- $\beta$  treatment results in the recruitment of Smads 3 and 4 to the previously identified TGF- $\beta$  Inhibitory Element (TIE) within the c-Myc promoter.
- The Smad3 MH1 domain specifically and directly binds to a core sequence 3' within the c-Myc TIE with a similar affinity as to which it binds to the SBE. This sequence (TGGCGGGA), termed a Repressive Smad Binding Element (RSBE), is distinct from an SBE (GTCT or AGAC).
- A mutation within the  $\beta$ -hairpin loop of the MH1 domain of Smad3 (R74A) disrupts its ability to bind to both the SBE and TIE/RSBE, suggesting this region of Smad3 binds to both sequences or the mutation disrupts proximal structure that mediates S3:TIE/RSBE interaction.
- SBE and TIE sequences exhibit distinct protein recognition as demonstrated by exclusive, direct binding of full length Smad4 to the SBE, but not the RSBE, and E2F-4 binding to the RSBE, yet not the SBE.
- The RSBE within the c-Myc promoter is both required and sufficient for TGF- $\beta$  induced transcriptional repression.
- Smad3 is recruited to the SBEs in the PAI-1 promoter and TIE/RSBE in the c-Myc promoter *in vivo*.

## Reportable outcomes

### Publications:

- 1) Wong, C., E.M Rougier-Chapman, J.P Frederick, M.B Datto, N.T. Liberati, J-M Li and X-F Wang. 1999. Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor  $\beta$ . *Mol. Cell Biol.* **19**:1821-1830. Attached in last annual report.
- 2) Liberati, N. T., M.B. Datto, J.P. Frederick, X. Shen, C. Wong, E.M. Rougier-Chapman and X-F Wang. 1999. Smads bind directly to the Jun family of AP-1 transcription factors. *Proc. Natl. Acad. Sci. USA* **96**:4844-4849. Attached in last annual report.
- 3) Shen, X., P.P. Hu, N.T. Liberati, M.B. Datto, J.P. Frederick, and X-F Wang. 1998. TGF- $\beta$ -induced phosphorylation of Smad3 regulates its interaction with coactivator p300/CREB-binding protein. *Mol. Biol. Cell* **9**:3309-3319. Attached in last annual report.
- 4) Datto, M.B.\*, J.P. Frederick\*, L. Pan, A.J. Borton, Y. Zhuang and X-F Wang. 1999. Targeted disruption of Smad3 reveals an essential role in transforming growth factor  $\beta$ -mediated signal transduction. *Mol. Cell. Biol.* **19**:2495-2504. Attached in last annual report.

\*Denotes equal contribution.

A manuscript describing the identification of c-Myc as a direct target for Smad mediated transcriptional repression and the analysis of TGF- $\beta$  induced repression of c-Myc is currently in preperation.

### Poster Presentations:

- 1) Frederick, J.P. 2000. *Department of Defense Breast Cancer Era of Hope Conference*. Smad3 is essential for transforming growth factor beta (TGF- $\beta$ ) mediated growth inhibition of primary fibroblasts and epithelial cells.
- 2) Annual Department and Graduate School research presentations.

### Speaker Presentations:

- 1) Frederick, J.P. 2000. *Department of Defense Breast Cancer Era of Hope Conference*. Smad3 is essential for transforming growth factor beta (TGF- $\beta$ ) mediated growth inhibition of primary fibroblasts and epithelial cells: Implications in breast and colon carcinogenesis.
- 2) Department retreat speaker.

## Conclusions

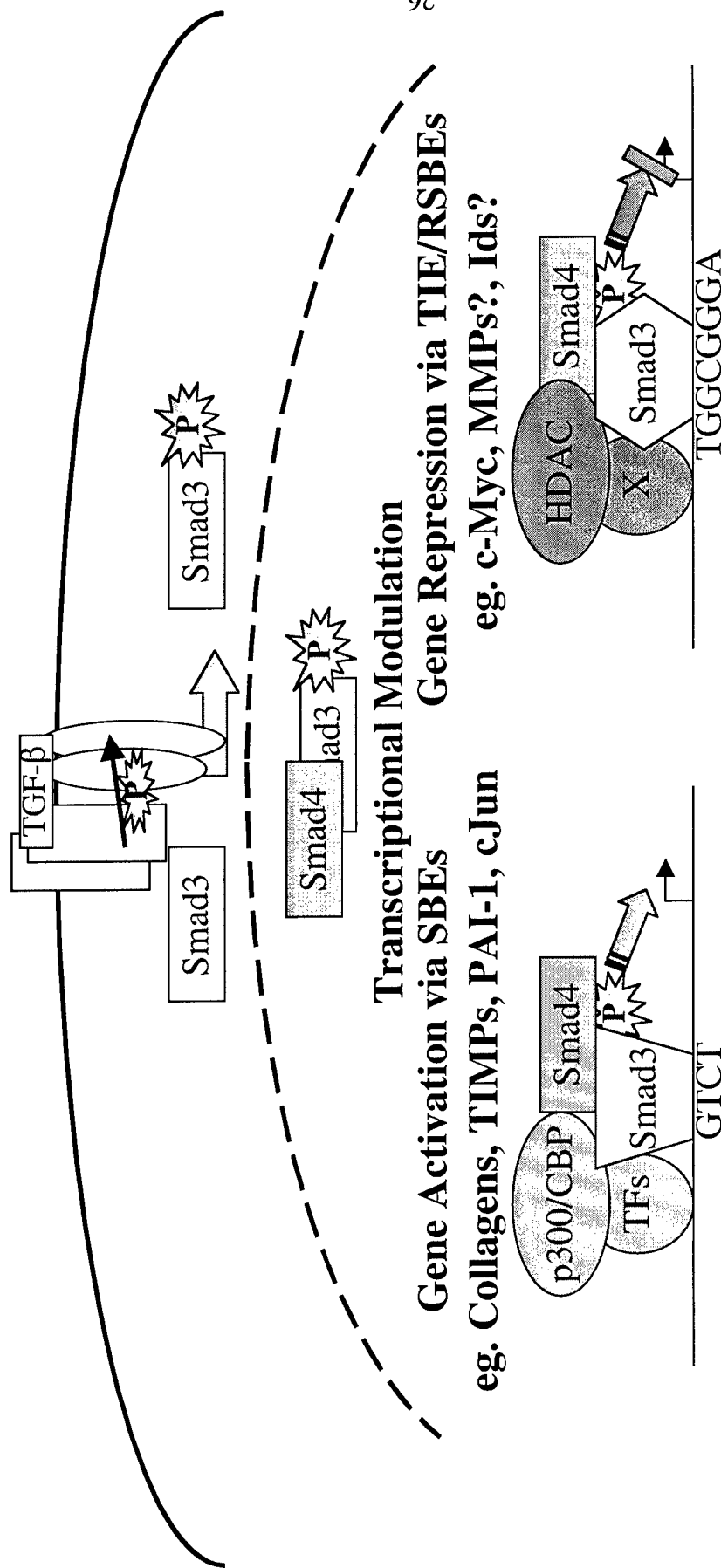
Transforming growth factor-beta (TGF- $\beta$ ) clearly plays a complex role in the physiology of mammary gland development and autocrine/paracrine homeostasis, and the pathophysiology of breast carcinogenesis. The ability of TGF- $\beta$  to potently suppress the proliferation of normal breast epithelial cells may be central to its putative role in tumor suppression of early stage breast cancers. However, it is important to note that in later stage breast cancers, in which epithelial cells are often refractory to the antiproliferative effects of TGF- $\beta$ , the modulation of the extracellular matrix (ECM) by the TGF- $\beta$  signal may contribute to breast cancer progression. Thus, the elucidation of the mechanisms by which TGF- $\beta$  is able to exert these effects is relevant to the further understanding of breast cancer initiation and possibly prevention.

The Smad family of proteins are the most well characterized mediators of the TGF- $\beta$  signal. The purpose of these studies was to further define the role of the Smads as TGF- $\beta$ -activated transcriptional regulators with particular attention to target genes involved in cellular growth inhibition, and thus, genes potentially involved in the tumor suppression of early breast cancers. The ability of the Smads to act as transcriptional activators via TGF- $\beta$  induced recruitment to Smad Binding Elements (SBE) within the promoters of TGF- $\beta$  target genes has been well established. However, the elucidation of the molecular mechanisms involved in TGF- $\beta$  mediated transcriptional repression of target genes suppressed by TGF- $\beta$  treatment are only now being uncovered. The proto-oncogene c-Myc is repressed by TGF- $\beta$  and this repression is paramount for the manifestation of TGF- $\beta$  mediated growth arrest of epithelial cells. We have shown that Smad3 is required for both TGF- $\beta$  induced c-Myc repression and subsequently growth arrest in epithelial cells. The transcriptional repression of c-Myc is dependent on Smad3 binding to a novel Smad binding element within the TGF- $\beta$  Inhibitory Element (TIE) of the c-Myc promoter, termed a Repressive Smad Binding Element (RSBE). Thus, we have established Smad3 as an essential component of TGF- $\beta$  induced growth inhibition and contributed to the understanding of how this growth arrest program is initiated. It is in this capacity of transducing the TGF- $\beta$  antiproliferative signal that Smad3 may act as a putative tumor suppressor in early stages of breast cancers.

It is known that the TGF- $\beta$  signaling pathway has a biphasic role in many cancers, in which it may be tumor suppressive in early stages through its antiproliferative effects yet promoting in later stages through its modulation of the ECM. The possibility that Smad mediated repression of target genes, such as c-Myc, involved in the TGF- $\beta$  mediated growth arrest program via RSBE binding and Smad mediated activation of target genes involved in ECM modulation, such as PAI-1, via SBE binding may be pharmacologically distinguishable could contribute to rationale, stage-dependent treatment of cancers.

Please see the next page for a model representation of Smad activity.

A model for how a single transcriptional modulator, Smad3, can confer both activating and repressive effects by directly binding to distinct sequences within oppositely regulated promoters.



-disparate sets of Smad3 binding partners based on cellular context, surrounding promoter elements and possibly distinct DNA-induced conformations of Smad3 upon SBE vs. TIE/RSBE binding

-possible to separate TGF- $\beta$  activated genes from those that are actively repressed-->Smad3 mutation or post-translational modification

Implications for the biphasic role of TGF- $\beta$  in multistage carcinogenesis.

## References

1. Barker, N., G. Huls, V. Korinek and H. Clevers. 1999. Restricted high level expression of Tcf-4 protein in intestinal and mammary gland epithelium. *Am. J. Path.* **154**:29-35.
2. Bonilla, M., M. Ramirez, J. Lopez-Cueto and P. Gariglio. 1988. In vivo amplification and rearrangement of the cMyc oncogene in human breast tumors. *J. Natl. Cancer Inst.* **80**:665-671.
3. Shi, Y. et al. 1998. Crystal structure of Smad MH1 domain bound to DNA: Insights on DNA binding in TGF- $\beta$  Signaling. *Cell*, **94**, 585-594.
4. Datto, M.B., J.P. Frederick, L. Pan, A.J. Borton, Y. Zhuang and X-F Wang. 1999. Targeted disruption of Smad3 reveals an essential role in transforming growth factor  $\beta$ -mediated signal transduction. *Mol. Cell. Biol.* **19**:2495-2504.
5. Dennler, S., S. Itoh, D. Vivien, P. ten Dijke, S. Huet and J-M Gauthier. 1998. Direct binding of Smad3 and Smad4 to critical TGF $\beta$ -inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO* **17**:3091-3100.
6. Escot, C., C. Theillet, R. Lidereau, F. Spyrtos, M.H. Champeme, J. Gest and R. Callahan. 1986. Genetic alteration of the cMYC protooncogene (MYC) in human primary breast carcinomas. *PNAS* **83**:4834-4838.
7. Chen, C-R, et al. Defective repression of c-Myc in breast cancer cells: A loss at the core of the TGF- $\beta$  growth arrest program. *PNAS*, **98**, 992-999.
8. Hua, X., X. Jiu, D.O. Ansari and H.F. Lodish. 1998. Synergistic cooperation of TGE3 and Smad proteins in TGF- $\beta$ -induced transcription of the plasminogen activator inhibitor-1 gene. *Genes Dev.* **12**:3084-3095.
9. Leder, A., P.K. Pattengale, A. Kuo, T.A. Stewart and P. Leder. 1986. Consequences of widespread deregulation of the c-myc gene in transgenic mice: multiple neoplasms and normal development. *Cell* **45**:485-495.
10. Liberati, N. T., M.B. Datto, J.P. Frederick, X. Shen, C. Wong, E.M. Rougier-Chapman and X-F Wang. 1999. Smads bind directly to the Jun family of AP-1 transcription factors. *Proc. Natl. Acad. Sci. USA* **96**:4844-4849.
11. Mariani-Constantini, R., C. Escot, C. Theillet, A. Gentile, G. Mero, R. Lidereau and R. Callahan. 1988. In situ cMyc expression and genomic status of the cMyc locus in infiltrating ductal carcinomas of the breast. *Cancer Res.* **48**:199-205.

12. Moser, A.R., E.M. Mattes, W.F. Dove, M.J. Lindstrom, J.D. Haag and M.N. Gould. 1993. APCmin, a mutation in the murine APC gene, predisposes to mammary carcinogenesis and focal alveolar hyperplasia. *PNAS* **90**:8977-8986.
13. Pennisi, E. 1998. How a growth control path takes a wrong turn to cancer. *Science* **281**:1438-1441.
14. Pierce, D.F., A.E. Gorska, A. Chytil, K.S. Meise, D.L. Page, R.J. Coffey, Jr. and H.L. Moses. 1995. Mammary tumor suppression by transforming growth factor  $\beta$ 1 transgene expression. *Proc. Natl. Acad. Sci. USA* **92**:4254-4258.
15. Pietenpol, J.A., J.T. Holt, R.W. Stein and H.L. Moses. 1990. Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. *Proc. Natl. Acad. Sci. USA* **87**:3758-3762.
16. Shen, X., P.P. Hu, N.T. Liberati, M.B. Datto, J.P. Frederick, and X-F Wang. 1998. TGF- $\beta$ -induced phosphorylation of Smad3 regulates its interaction with coactivator p300/CREB-binding protein. *Mol. Biol. Cell.* **9**:3309-3319.
17. Shi Y., Y-F Wang, L. Jayaraman, H. Yang, J. Massague and N.P Pavletich. 1998. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF- $\beta$  signaling. *Cell* **94**:585-594.
18. Song C-Z, T.E. Siok and T.D. Gelehrter. 1998. Smad4/DPC4 and Smad3 mediate transforming growth factor-  $\beta$  (TGF-  $\beta$ ) signaling through direct binding to a novel TGF-  $\beta$ -responsive element in the human plasminogen activator inhibitor-1 promoter. *J. Biol. Chem.* **273**:29278-29290.
19. Stewart, T.A., P.K. Pattengale and P. Leder 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MMTV/myc fusion genes. *Cell* **38**:627-637.
20. Stroschein, S.L., W. Wang and K. Luo. 1999. Cooperative binding of Smad proteins to two adjacent DNA elements in the plasminogen activator inhibitor-1 promoter mediates transforming growth factor  $\beta$ -induced Smad-dependent transcriptional activation. *J. Biol. Chem.* **274**:9431-9441.
21. Wong, C., E.M Rougier-Chapman, J.P Frederick, M.B Datto, N.T. Liberati, J-M Li and X-F Wang. 1999. Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor  $\beta$ . *Mol. Cell Biol.* **19**:1821-1830.
22. Yingling, J.M., M.B. Datto, C. Wong, J.P. Frederick, N.T. Liberati and X-F Wang. 1997. Tumor suppressor Smad4 is a transforming growth factor  $\beta$ -inducible DNA binding protein. *Mol. Cell. Biol.* **17**:7019-7028.

23. Zawal, L., J.L. Dai, P. Buckhaults, S. Zhou, K.W. Kinzler, B. Vogelstein and S.E. Kern. 1998. Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol. Cell.* **1**:611-617.
24. Zhang, Y., X-H Feng, R-Y Wu, and R. Derynck. 1996. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* **383**:168-172.
25. Nishita, M., et al. 2000. Interaction between Wnt and TGF-beta signaling pathways during formation of Spemann's organizer. *Nature* **403**: 781-785.
26. Lin, S.-Y., et al. 2000. Beta-catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression. *PNAS* **97**: 4262-4266.
27. Takaku, K., et al. 1998. Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* **92**: 645-656.
28. He, T.C., et al. 1998. Identification of c-Myc as a target of the APC pathway. *Science* **281**: 1509-1512.
29. Tetsu, O. and McCormick F. 1999. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**: 422-426.
30. Shtutman, M., et al. 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *PNAS* **96**: 5522-5527.
31. Alexandrow, M.G., et al. 1995. Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor beta 1. *PNAS*, **92**: 3239-3243.
32. Claasen, G.F., et al. 2000. A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest. *PNAS*, **97**, 9498-9503.
33. Pietenpol, J.A., et al. 1990. Transforming growth factor beta1 suppression of c-Myc gene transcription: Role in inhibition of keratinocyte proliferation. *PNAS*, **87**, 3758-4762.
34. Pietenpol, J.A., et al. 1991. Factor binding element in the human c-Myc promoter involved in transcriptional regulation by TGF-beta1 and by the retinoblastoma gene product. *PNAS*, **88**, 10227-10231.



DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

8 Jan 2003

MEMORANDUM FOR Administrator, Defense Technical Information  
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,  
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed. Request the limited distribution statement for the enclosed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management

ADB265840  
ADB279138  
ADB264578  
ADB281679  
ADB281645  
ADB261128  
ADB261339  
ADB273096  
ADB281681  
ADB259637  
ADB256645  
ADB262441  
ADB281674  
ADB281771  
ADB281612

ADB266633  
ADB251763  
ADB281601  
ADB258874  
ADB281773  
ADB281660  
ADB259064  
ADB266141  
ADB281664  
ADB258830  
ADB266029  
ADB281668  
ADB259834  
ADB266075  
ADB281661

ADB282069  
ADB265386  
ADB282057  
ADB258251  
ADB264541  
ADB241630  
ADB281924  
ADB281663  
ADB281659